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Retention of enzyme activity following freeze-drying the mycelium of ectomycorrhizal isolates

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Summary

Qualitative enzyme assays were used as a tool to investigate the stability of freeze-dried mycorrhizal fungi. Both lyophilized (L) and non-lyophilized (NL) mycelia of individual isolates showed identical response for all the enzymes tested (nitrate reductase, protease, pectinase, and nuclease). All the isolates showed positive nitrate reductase activity, except two isolates of *Thelephora terrestris* (both L and NL). Both L and NL cultures of individual isolates showed substrate specificity (between gelatin and casein) for protease activity. Though both L and NL mycelia of all the culture isolates grew upon pectin substrate, there was no pectinase activity expressed. RNAase activity was variously exhibited (little activity, little growth–no activity, and no growth–no activity) by individual test cultures. The consistencies in growth and enzyme activity of the cultures before and after lyophilization imply the stability of the freeze-dried vegetative mycelium.

Introduction

The emphatic statement of Hawksworth (1991) saying "it is regrettable that the conservation of fungi has received scant attention in spite of its role in ecosystem function, maintenance of biodiversity, and the largely unexplored genetic resource they represent", speaks for itself the need to preserve the rich resource of diverse and valuable fungi. Mycorrhiza is one amongst the current topics of biological research with many open questions. The serial subculture method followed for storing mycorrhizal germ plasm has encountered problems such as decrease in efficacy or vigour (Giltrap 1981; Marx 1981). Ectomycorrhizal fungi grow as mycelial filaments upon artificial substrate and completely lack the ability to sporulate *in vitro* (Kowalski 1974). Mycelial non-sporulating fungi were reported to be non-lyophilizable (Simione & Brown 1991; Smith & Onions 1994). Lyophilization of vegetative mycelium of ectomycorrhizal fungi was attempted and an optimized protocol was developed (Sundari & Adholeya 1999). Once the viability of the organism subjected to freeze-drying is tested, the next most important objective is to test the functional viability/stability. Functional viability is the ability of a treated sample to exhibit a specific function or functions expressed as a proportion of the same function exhibited by the sample before treatment or by an identical fresh untreated sample (Abbott 1989; Pegg 1989). To assess the quality of freeze-dried product, viability assays to test physiological growth, metabolic activity and symbiotic ability were performed (Sundari 1999) with freeze-dried cultures. All the freeze-dried culture isolates succeeded in establishing synthesis with either or both hosts *Eucalyptus*/*Pinus*. Total biomass estimation and total protein content also showed similarity in expression for both lyophilized and non-lyophilized vegetative mycelia of every species tested (Sundari 1999).

Qualitative enzyme assays were used in the present study as a tool to study the functional stability of the freeze-dried ectomycorrhizal mycelium. The test for response of cultures on enzyme-specific substrates in defined and semi-defined media is a simple and fast method to study a variety of fungal enzymatic activities (Pegg 1989; Paterson & Bridge 1994). The presence of few enzymes that form the fundamental units of metabolism was tested both before and after lyophilization. The enzymes selected in the present study were, nitrate reductase, protease, pectinase and nuclease. These enzymes are functionally important either in nutrient mobilization or active metabolism of mycorrhizal fungus (Ramsteldt & Söderhäll 1983; Duddridge & Read 1984; Abuzinadah & Read 1986; Ho 1987; Hutchison & Malloch 1988; Guttenberger 1995; Colpaert & Van Laere 1996). The assays have helped to show the retention of enzyme activity in freeze-dried mycorrhizal fungi.

Materials and Methods

Fungal isolates

The species *Laccaria fraterna* (EM-1083), *Laccaria laccata* (Scopp.:Fr.) Berk. & Broome (EM-1105), *Laccaria amethystina* (EM-1091), *Amanita muscaria* (L.:Fr.) Pers. (EM-1060), *Thelephora terrestris* (Ehrh.) Fr. (EM-1247), *T. terrestris* (EM-1077), *Scleroderma cepa* Pers. (EM-1233), were subjected to freeze drying using appropriate cryoprotectant (Sundari 1999) according to the method of Sundari & Adholeya (1999). Three replicate tubes with protectant (per isolate) were subjected to lyophilization, while each replicate tube contained three mycelial agar discs. The cultures were pre-frozen (to $-30 \pm 2\,^{\circ}C$) before subjecting to lyophilization so as to ensure minimum injury to mycelium during subsequent stages. The cultures were dried under vacuum at a temperature of $100 \pm 2\,^{\circ}C$. Extended drying of the cultures after attaining the end point was avoided to overcome damage due to over-drying. The lyophilized cultures were rehydrated for a minimum of 10 h before plating on MMN agar (Molina & Palmer 1982). The cultures were incubated in a BOD at $22 \pm 2\,^{\circ}C$. The experiment was repeated three times to check the consistency of the results.

The radial growth of the lyophilized, reconstituted colony was measured using the software Quantimet C+ (Leica, Switzerland) and compared with that of the culture growth before subjecting to lyophilization. The results were analysed using the software Coplot/Costat.

Test for assimilation of nitrate nitrogen

The nitrogen assimilation medium contained sodium nitrate added at the rate of 0.2 g per 100 ml of basal medium (Paterson & Bridge 1994). The pH of the medium was adjusted to 5.6, before autoclaving and poured into 90-mm petri plates (25 ml/plate).

Test for assimilation of protein

Method 1
To the basal medium, 15% skim milk was added (Paterson & Bridge 1994). The pH was set to 5.4 before autoclaving at 5 psi for 30 min. The cooled medium was poured into 90-mm petri plates (25 ml/plate).

Method 2
Basal medium was prepared without glucose and warmed to 50–60 °C (Paterson & Bridge 1994). Gelatin was slowly added in small amounts until it was completely dissolved (120 g/l). The warm medium was dispensed into glass bottles at the rate of 30 ml/bottle, and autoclaved at 10 psi for 10 min. Test media were chilled (4 °C for 30 min) after the incubation period and before the interpretation of results.

Test for utilization of pectin

Citrus pectin was provided as sole carbon source (1 g/l), and ruthenium red was included in the medium at a concentration of 150 mg/l (Paterson & Bridge 1994). The medium was autoclaved at 5 psi for 20 min and poured into 90-mm petri plates (25 ml/plate).

Test for utilization of free nucleic acids

The test medium was an ammonium salt medium with RNA as main carbon source, supplied at the rate of 2 g/l (Paterson & Bridge 1994). The medium was autoclaved at 10 psi for 10 min, and poured into 90-mm petri plates (25 ml/plate). After incubation and growth, the plates were flooded with a weak acid solution (1 M HCl) to precipitate unused nucleic acid.

All the agar-based enzyme tests were carried out with both the non-lyophilized (NL) and lyophilized (L) cultures (taken from a fully-grown lyophilized, reconstituted colony) isolates at the rate of three replicates per enzyme per isolate. The test media were all centrally inoculated with a culture disc cut from the growing edges of colony. Cultures of same age (4 weeks) and inoculum disc of same size (0.69 cm^2) were taken. All the experimental plates were incubated in BOD at $22 \pm 2\,^{\circ}C$.

Results and Discussion

All the cultures sustained freeze drying with every culture showing revival in all the nine agar discs subjected to lyophilization, except the culture *S. cepa* where seven out of nine discs showed revival. The freeze-dried culture isolates, showed a lag period after rehydration, which varied from isolate to isolate (Table 1). This is a common observation in freeze-dried systems. Though the freeze-dried cultures showed a lag phase before growth initiation after reconstitution, their subsequent growth was comparable to that of the non-lyophilized cultures (Table 1). Faster growth and enhanced development in re-cultivated lyophilized specimens was reported earlier (Tan & Stalpers 1991).

All the cultures tested, except for the slow growing isolate of *T. terrestris*, showed positive nitrate reductase activity (Table 2). A reduction in the intensity of medium color (from brownish yellow to lighter tones of yellow) was observed in active producers upon incubation. There was a uniform trend between isolates of the same species (positive growth and no activity in both *T. terrestris* isolates), and amongst species of same genera (positive in all three *Laccaria* species). This supports the earlier opinion (Hutchison & Malloch 1988) that species within a genus share similar reactions and thus make themselves unique with a set of cultural characters. Similar results were obtained both with NL and L mycelial cultures for all the isolates.

Table 1. Growth of freeze-dried cultures compared with that of respective non-freeze-dried isolates.

Name of the culture	Isolate number	Lag phase (in days)	Radial growth in non-lyophilized/ lyophilized culture (area in cm^2)	LSD at 0.01 level of significance	Correlation coefficient (*r*-value)
Laccaria laccata	EM-1105	3	59.42/57.51	1.05	0.998
L. fraterna	EM-1083	4	58.37/58.01	0.94	0.996
L. amethystina	EM-1091	8	25.58/25.64	0.713	0.998
Amanita muscaria	EM-1060	4	56.53/56.22	1.272	0.996
Thelephora terrestris	EM-1247	4	59.17/59.35	0.866	0.99
T. terrestris	EM-1077	15	59.01/58.80	0.85	0.997
Scleroderma cepa	EM-1233	11	18.45/18.94	0.644	0.995

Table 2. Enzyme activities expressed by culture isolates upon enzyme specific substrates.

Name of the culture	Isolate number	Nitrate reductase	Protease casein	Protease gelatin	Nuclease	Pectinase
Laccaria laccata – non-lyophilized	EM-1105	+ + +	+ + +	+ + + +	+	–
*L. laccat*a – lyophilized		+ + +	+ + +	+ + + +	+	–
L. fraterna – non-lyophilized	EM-1083	+ + +	+ + +	+ + +	+	–
L. fraterna – lyophilized		+ + +	+ + +	+ + +	+	–
L. amethystina – non-lyophilized	EM-1091	+ + +	+ + + +	+	–	–
L. amethystina – lyophilized		+ + +	+ + + +	+	–	–
Amanita muscaria – non-lyophilized	EM-1060	+ + +	–	+ + +	–	–
A. muscaria – lyophilized		+ + +	–	+ + +	–	–
Thelephora terrestris – non-lyophilized	EM-1247	–	–	+ + + +	–	–
T. terrestris – lyophilized		–	–	+ + + +	–	–
T. terrestris – non-lyophilized	EM-1077	–	+ + + +	–	–	–
T. terrestris – lyophilized		–	+ + + +	–	–	–
Scleroderma cepa – non-lyophilized	EM-1233	+ + +	+ + + +	+ +	–	–
S. cepa – lyophilized		+ + +	+ + + +	+ +	–	–

+ + + +: Activity surpasses colony spread; + + +: activity same as colony spread; + +: activity less than colony spread; +: very little/ negligible activity; –: no detectable activity.

Most of the isolates tested hydrolysed casein and/or gelatin implying protease activity (Table 2). Hydrolysis of casein resulted in a clear zone around the colony and hydrolysis of gelatin led to liquefaction of the medium. Species of *Laccaria* and *Scleroderma* showed activity on both the substrates while others showed substrate specificity. Specificity was observed among isolates (*T. terrestris*), between species (*L. laccata*/*L. fraterna* and *L. amethystina*) and between genera (*Laccaria* and *Amanita*). *Amanita muscaria* showed delayed activity (liquefaction took 2 weeks on gelatin substrate). Each lyophilized mycelial culture responded in exactly the same way as the original non-lyophilized culture.

The present study suggests complete absence of pectinases in all of the isolates tested though all the cultures grew moderately well on the substrate (Table 2). Similar results were obtained with both lyophilized and non-lyophilized culture isolates. There are contradicting reports as to the presence of pectinolytic enzymes in mycorrhizal fungi (Lindeberg & Lindeberg 1977; Giltrap & Lewis 1982; Ramsteldt & Söderhäll 1983; Duddridge & Read 1984; Hutchinson & Malloch 1988). Either catabolite repression or product inhibition might occur in mycorrhizal fungi, leading to negligible presence or complete absence of pectinases (Gilltrap & Lewis 1982), due to the symbiotic nature of mycorrhizal fungi. On the other hand, as enzyme activity is substrate-specific, the absence of pectinases might as well be due to either non-provision of the appropriate substrate, or unfavorable medium pH (Read 1991). Also, the near absence of nucleases in mycorrhizal fungi tested in the present study demonstrates the adaptation of these fungi to the symbiotic association.

A clear zone around the colony indicates nucleic acid hydrolysis. Two species of *Laccaria* grew upon the substrate and showed slight nuclease activity indicated by a faint decrease in the opacity of the medium. *S. cleroderma cepa* and *T. terrestris* (EM-1077) failed to grow on the substrate even after 6 weeks of incubation. The remaining cultures grew upon the substrate but failed to show any activity. Lyophilized and non-lyophilized cultures responded similarly towards RNA for all isolates tested.

The similarity of mycelial cultures with respect to response towards the aforesaid enzyme substrates before and after lyophilization suggests stability of enzymatic activity in mycorrhizal fungi during lyophilization.

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